

AMENDMENTS TO THE SPECIFICATION:

Please delete the paragraph on page 2, line 32 to page 3, line 2 and replace it with the following paragraph:

It is therefore an object of the present invention a nucleic acid encoding for a protein with endoribonucleasic activity which is polyU sequences and single filament specific, Mn^{++} -dependent and releases as cleavage products molecules having 2'-3' cyclic phosphate and 5'OH ends. Preferably nucleic acid includes substantially ~~SEQ ID No. 1~~ SEQ ID NO: 1 nucleotide sequence, functional homologs thereof or complementary sequence thereto.

Please delete the paragraph on page 3, lines 10-23 and replace it with the following paragraph:

It is a further object of the invention a protein with endoribonucleasic activity which is poly-U sequences and single filament specific, Mn^{++} -dependent and releases RNA molecules bearing 2',3' cyclic phosphate and 5'OH ends as cleavage products, or functional portions thereof. Preferably the protein is encoded by inventive ~~SEQ ID No. 1~~ SEQ ID NO: 1 nucleic acid, more preferably protein substantially has ~~SEQ ID No. 2~~ SEQ ID NO: 2 amino acid sequence. Advantageously the protein is produced by synthetic or recombinant route using

methods known by those skilled in the art. It is a further object of the present invention the use of the protein with endoribonucleasic activity in analytical or synthetic applications. Particularly analytical applications can be selected from the group including RNA sequencing, point mutation detection, RNA molecular digital fingerprinting determination, RNA structural analysis, Rnase protection assays.

Before the paragraph beginning at page 4, line 4, insert the following heading:

--BRIEF DESCRIPTION OF THE DRAWINGS--.

Please delete the paragraph on page 4, line 33 to page 5, line 14 and replace it with the following paragraph:

Figure 3 shows that the XendoU activity is U-specific and produces 2'-3' cyclic phosphate. A, ³²P ATP labelled synthetic oligoribonucleotide PI, ~~SEQ ID No 3~~ SEQ ID NO: 3, containing the distal cleavage site upstream from U16, and its mutant derivatives (P2, ~~SEQ ID No 4~~ SEQ ID NO: 4; P3, ~~SE ID No 5~~ SEQ ID NO: 5) were incubated with the unfractionated extract (lanes 2) or with purified XendoU (lanes 3), under standard conditions for 30 min. RNA molecules were extracted and analysed on 10% polyacrylamide-7M urea gel. In lanes 1 untreated RNA is shown, in lane M RNA marker generated by

alkaline digestion of P1 (~~SEQ ID No 3~~ SEQ ID NO: 3) is shown; arrows indicate cleavage sites. On the right side the sequences of the oligoribonucleotides are reported. B, The ³²P-labelled 1-1 molecules, schematically represented on the left side, generated by incubation of U16-containing precursor in ONE (ONE), with purified XendoU (XendoU) or after injection in oocytes (in vivo), were gel purified, and their 3' end analysed. The molecules were incubated with 1 unit of alkaline phosphatase (lane 1) or with 10 mM HCl (lanes 2) or with alkaline phosphatase after acid treatment (lanes 3). After incubation the RNAs extracts were analysed by electrophoresis on 10 % polyacrylamide-7M urea gel. Untreated molecules were run as control in lanes 4.

Please delete the paragraph on page 5, lines 15-23 and replace it with the following paragraph:

Figure 4 shows cDNA (SEQ ID NO: 1) and amino acid sequence (SEQ ID NO: 2) of XendoU. Nucleotides of the 5' and 3' untranslated regions are shown in small letters, nucleotides of the ORF in capital letters. Above each codon the corresponding amino acid is shown (~~SEQ ID No 1~~). The sequence portions determined by automated Edman degradation and mass mapping experiments (see "Experimental Procedures") are indicated by numbers 1, 2 and 3. The stop codon is identified by an asterisk. Numbers on the right side of

diagram correspond to the amino acid residues. Underlined are the amino acid sequences identified by MALDI-mapping experiments.

Please delete the paragraph on page 5, line 34 to page 6, line 18 and replace it with the following paragraph:

Figure 6 shows that XendoU is involved in U86 snoRNA biosynthesis. A, U86 processing is analysed in vivo by injection of ^{32}P -labelled U86-containing precursor (P) in *X. laevis* oocytes (lanes in vivo), or in vitro by incubation of the RNA precursor in ONE (ONE), or with purified XendoU (XendoU). The numbers below indicate different incubation times: 0 min (lane 1), 10 min (lanes 2), 45 min (lanes 3), 3 hours (lanes 4), 16 hours (lanes 5). RNA was then extracted and loaded on a 6% polyacrilamide-7M urea gel. The processing products are schematised aside. Arrows indicate specific XendoU cleavages. B, ^{32}P -labelled UhindIII primer, depicted below, was reacted with unlabelled 1-4 molecules obtained after 10 min of incubation in oocytes (lane in vivo), 45 min of incubation in ONE (ONE) or 45 min of incubation with purified XendoU (XendoU). The products of primer extension were run in parallel with the sequence (lane G, A, T, and C) performed with the same oligonucleotide on U86. The sequence is reported on the left side (SEQ ID NO: 10): the arrow points to the XendoU cleavage sites. C, U86-containing precursor was

incubated, in the presence of Mn^{++} ions, in ONE (ONE), with XendoU produced by reticulocyte lysate (ret-XendoU/+Mn), or with reticulocyte lysate as such (lanes ret/+Mn). As control, pre-mRNA was incubated with XendoU produced by reticulocyte lysate in the absence of Mn^{++} ions (ret-XendoU/-Mn). The numbers below indicate incubation times: 0 min (lane 1), 45 min (lanes 2).

Please replace the paragraph beginning at page 6, line 27, with the following rewritten paragraph:

XendoU was purified from oocyte nuclear extracts (ONE). ONE was fractionated by ammonium sulphate precipitation. Solid ammonium sulphate (280 mg/ml) was slowly added to the nuclear extract up to 45% saturation and the suspension was stirred for 30' at 4°C and then centrifuged at 12.000 rpm for 30' at 4°C. The supernatant was made 70% saturated by a further addition of ammonium sulphate (240 mg/ml). The suspension was stirred and centrifuged as above. The resulting pellet was dissolved in ONE buffer (25 mM Hepes pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) and then applied onto an hydroxyapatite column (CHT-II Econocolumn, Biorad). Column was washed with ONE buffer and then eluted with 5 column volumes of 100 mM Na-phosphate pH 7 in ONE buffer. The eluate was collected in 1 ml fractions next tested for the endonuclease activity. Selected fractions were pooled, diluted with 3

volumes of ONE buffer and applied on a Blue Sepharose column (Blue Sepharose Fast Flow Pharmacia). The column was washed with ONE buffer and then eluted with 5 column volumes of 0.2 M NaCl in ONE buffer. The eluate was then collected in 1 ml fractions; those displaying the specific activity were pooled and dialysed against ONE buffer. Protein mixture was subjected to a second fractionation on hydroxyapatite column. The elution was performed with 10 column volumes of a linear gradient 0-100 mM Na-phosphate pH 7 in ONE buffer. Collected fractions were tested and those with activity were pooled and concentrated by means of ultrafiltration device (~~Centricon~~ CENTRICOMTM C10, Millipore). The concentrated fractions were then applied on a gel-filtration column (Pharmacia) previously equilibrated in ONE buffer. Elution was monitored collecting 0.5 ml fractions which were tested for specific activity.

Please replace the paragraph beginning at page 7, line 22, with the following rewritten paragraph:

Protein bands from SDS-PAGE analysis (5 µg) stained with Coomassie Blue R250 were excised, reduced with dithiothreitol and carboxamidomethylated. Gel pieces were equilibrated in 25 mM NH₄HCO₃, pH 8 and finally digested in situ with trypsin at 37 °C for 18 h. Peptides were extracted by sonication with 100 µl of 25 mM NH₄HCO₃/acetonitrile 1:1 v/v, pH 8 (twice). Peptide mixture was fractionated by reverse-phase HPLC on a ~~Vydac~~

VYDACTM C₁₈ column 218TP52 (250 x 1 mm), 5 µm, 300 Å pore size (The Separation Group, USA) by using a linear gradient from 5% to 60% of acetonitrile in 0.1% TFA over 60 min, at flow rate of 90 µL/min. Individual components were manually collected and lyophilised.

Please replace the paragraph beginning at page 7, line 33, with the following rewritten paragraph:

Sequence analysis was performed using a ~~Preecise~~ PROCISETM 491 protein sequencer (Applied Biosystems, USA) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems, USA) for the automated identification of PTH-amino acids.

Please delete the paragraph on page 8, lines 24-36 and replace it with the following paragraph:

The oligoribonucleotides

P1 (5'-GGAAACGUAUCCUUUGGGAG-3'), ~~SEQ ID No 3~~ SEQ ID NO:
3;

P2 (5'-GGAAACGUAUCCUUGGGAGTG-3'), ~~SEQ ID No 4~~ SEQ ID NO:
4;

P3 (5'-GGAAACGUAUCCUCUGGGAG-3'), ~~SEQ ID No 5~~ SEQ ID NO:
5;

P4 (5'-GGAAACGUAUCCUGUGGGAG-3'), ~~SEQ ID No 6~~ SEQ ID NO:
6;

Please delete the paragraph on page 9, lines 15-20 and replace it with the following paragraph:

A X. laevis stage 28 embryo cDNA library, constructed in λ ZAP II vector, was screened using a specific probe obtained by PCR amplification on X.laevis cDNA with degenerated oligonucleotides (MAHs 5'-ATGGCICAYGAYTAYYTIGT-3', ~~SEQ ID No 7~~ SEQ ID NO: 7 and IGTA 5'-ACIGGRTAIGCIGTICCIAT-3', ~~SEQ ID No 8~~ SEQ ID NO: 8) designed on peptides obtained by tryptic digestion of purified XendoU.

Please replace the paragraph beginning at page 9, line 22, with the following rewritten paragraph:

XendoU Open Reading Frame (ORF) was cloned into ~~Blue-Script~~ BLUE SCRIPTTM vector and ³⁵(S)Methionine-labelled protein was produced by in vitro transcription and translation using the TnT-coupled Reticulocyte Lysate System Kit (PROMEGATM) according to the manufacturer's instructions. Translational products were analysed on 10% SDS-PAGE.

Please delete the paragraph on page 9, lines 28-37 and replace it with the following paragraph:

In vitro transcribed U86-containing precursor was obtained from a standard T7 reaction, but in the presence of 500 μ M unlabelled UTP. The transcript was injected into X.

laevis oocytes or incubated in ONE or with purified XendoU. The processing product I-4 RNA was gel-purified and reverse transcribed (SS pre-amplification system - GIBCO) with the 5' terminally labelled oligonucleotide UHindIII (5'-AAGCTTCTTCATGGCGGCTCGGCCAAT-3', ~~SEQ ID No 9~~ SEQ ID NO: 9) complementary to 19 nucleotides at the 3' end of the downstream exon. The elongated products were run in parallel with the sequence obtained with the same primer on U86-containing precursor.

Please delete the paragraph on page 11, lines 3-9 and replace it with the following paragraph:

The substrate selectivity of XendoU was further addressed by incubating the purified enzyme with synthetic oligoribonucleotide (P1, ~~SEQ ID No 3~~ SEQ ID NO: 3), containing the distal XendoU cleavage site (site d, Figure 1A), localised upstream from U16, and with mutated derivatives thereof (P2, ~~SEQ ID No 4~~ SEQ ID NO: 4 and P3, ~~SEQ ID No 5~~ SEQ ID NO: 5). The obtained results, shown in Figure 3A, indicate that XendoU displays the same selectivity observed in vivo and that the minimal consensus cleavage site is an uracyl dimer.

Please delete the paragraph on page 11, line 36 to page 12, line 22 and replace it with the following paragraph:

After purification, protein samples from SDS-PAGE were reduced, alkylated and digested with trypsin as reported in the experimental section. The resulting peptide mixture was resolved by R-HPLC and selected peptide fractions were submitted to automated Edman degradation. The three sequence portions determined are reported in Figure 4 (indicated as 1, 2 and 3). From these amino acid sequences, degenerated oligonucleotides were derived and employed in different combinations and different orientation in PCR amplification reactions on cDNA from polyA⁺ RNA extracted from *X. laevis* oocytes. Only the reaction performed with sequence 1 (forward) and sequence 3 (reverse) resulted in an amplification product (500 bp). Sequencing of this product indicated the presence of an Open Reading Frame containing peptide 2. This cDNA probe was then utilised for the screening of a *X. laevis* stage 28 embryo cDNA library, allowing the isolation of a full-length cDNA (~~SEQ ID No 1~~ SEQ ID NO: 1). The amino acid sequence determined was confirmed by MALDI-MS spectra of the tryptic peptides. In fact, signals observed at m/z 565.29, 814.45, 1004.48, 10025.54, 1132.59, 1190.60, 1490.78, 1504.80, 1520.70, 1729.91, 1758.82, 1988.08, 2000.00, 2014.01, 2076.99, 2162.98, 2234.14, 2238.05, 2394.15, 2432.26, 3058.51 and 3370.66 were ascribed to peptides 196-200, 126-132, 117-124, 6-14, 117-125, 41-52, 114-125, 260-271, 15-26, 137-149, 53-67, 256-271, 53-69, 275-292, 204-220, 150-169, 133-149, 171-188,

170-188, 117-136, 6-31 and Ac4-31. This result allowed to cover 65% of the entire sequence, explaining the reluctance of a blotted protein sample to Edman degradation.